the viral NP and the cellular NPI-1 during influenza A virus infection.

7. Example: THE IDENTIFICATION OF NS1I-1 AND ITS INTERACTION WITH INFLUENZA NUCLEOPROTEIN NS1

In the example described below, the yeast interactive trap system was used to identify a human protein, NS1I-1 (NS1-interactor-1), from a HeLa cell cDNA library on the basis of its binding to NS1 of influenza A virus. NS1I-1 is shown herein to be recognized not only by NS1 proteins from five human and avian influenza A strains, but also by NS1 of influenza B virus. Surprisingly, NS1I-1 is homologous to a steroid dehydrogenase isolated from pigs (Leenders, et al., 1994, Eur. J. Biochem. 222: 221-227). Several proteins with a dehydrogenase function have recently been shown not only to have enzymatic activity but also to be involved in post-transcriptional events of gene-expression (Hentze, 1994, *supra*). This strong conservation supports an important functional role of the NS1I-1 interaction during the viral life cycle.

7.1. MATERIALS AND METHODS

7.1.1. YEAST, *E.COLI* STRAINS, AND PLASMIDS

Manipulations of nucleic acids, *Escherichia coli* and yeast followed essentially standard procedures as described elsewhere (Ausubel, et al., 1992, Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The yeast strains EGY40 (*Mata trp1 ura3 his3*) and EGY48 (*Mata trp1 ura3 his3 LEU2::pLEX-Aop6-LEU2*) as well as plasmids pEG202, pRFHM1, and pSH18-34, and the HeLa cell cDNA constructed in pJG4-5

have been described (Gyuris, et al., 1993, *supra*; Zervos, et al., 1993, *supra*). *E. coli* strains used for cloning and expression were MH3 (trpC araD lacX hsdR galU galK), DH5α (F⁻Φ80dlacZΔM15 Δ(lacZY-argF)U169 deoR recA1 endA1 hsdR17($r_K^-m_K^+$) supE44λ-thi- gyrA96 relA1), and BL26 (F⁻ompT hsdS_B($r_B^-m_B^-$) gal dcm). pLexA-NS1 was constructed by subcloning the cDNA of the NS segment of influenza virus A/PR/8/34 downstream of the LexA gene in pEG202. pGEX-NS1I-1 was constructed by subcloning the HeLa cDNA-insert of library plasmid pK5 as an EcoRI/XboI-fragment into pGEX-5X-1 (Pharmacia). DNA-oligonucleotides used were: GSP-I, 5′-dTCCTGATGTTGCTGTAGACG-3′,
GSP-II, 5′-dGCACGACTAGTATGATTTGC-3′, and the 5′RACE anchor primer (BRL), 5′-dCUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′

7.1.2. IDENTIFICATION OF NS1-INTERACTORS

The interactive trap selection was performed essentially as described for NPI-1 in Section 6.1.2, above. The selection strain was constructed by transforming EGY48 with the bait plasmid pLexA-NS1 and the lacZ-reporter plasmid pSH18-34. Expression of lacZ from pSH18-34 is transcriptionally controlled by a GAL1 promoter and LexA-dependent operator sites. A HeLa cell cDNA library was introduced into the selection strain using the lithium acetate method (Ito, et al., 1983, *supra*). Primary transformants were selected on trp⁻his⁻ura⁻ glucose plates. 1 x 10^6 cells representing 3.3 x 10^5 independent transformants were plated on 150 mm trp⁻his⁻ura⁻leu⁻-galactose plates to select for clones expressing NS1-interacting proteins. Viable cells were replica-transferred to a nitrocellulose filter and assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) as described (Ausubel et al.,

1992, *supra*). Positive clones were tested in a second round of selection by replica plating onto X-gal trp$^-$ his$^-$ura$^-$ galactose plates. Plasmid DNA was isolated from yeast clones expressing β-galactosidase activity only on galactose plates and library plasmids were recovered by transformation into *E.coli* MH3 as described in Section 6.1.2, above. The specificity of the isolated plasmids was tested by co-transformation with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses an unrelated LexA-bicoid fusion protein. The resulting strains were assayed for β-galactosidase activity on X-gal trp$^-$his$^-$ura$^-$ plates containing glucose or galactose. Plasmids that induced β-galactosidase only in the presence of galactose and only in conjunction with pLexA-NS1 were considered to encode true interacting proteins.

7.1.3. CLONING OF NS1I-1 5'-END CDNA

Cloning of cDNA derived from the 5'-end of NS1I-1 mRNA followed a RACE-procedure (rapid amplification of cDNA ends) (Frohmanm, et al., 1988, *supra*) using a 5'RACE-kit (BRL). First strand cDNA was synthesized from 1 μg of HeLa cell poly(A)-RNA hybridized to 2.5 pmol NS1I-1-specific oligonucleotide GSP-I using reverse transcriptase. The cDNA was tailed at the 5'-end with dC by terminal transferase. The product was used as a template for the amplification of a 5'RACE-product by PCR using a nested oligonucleotide GSP-II and an anchor primer provided by the kit. The resulting fragment was subcloned in pGEM-T (Promega) to form pRACENS1I-1, and sequenced by the standard dideoxy method. The NCBI-search was conducted using Fasta, Tfasta. Sequence comparison was conducted using Bestfit.

7.1.4. NORTHERN BLOT ANALYSIS

1 µg of HeLa cell poly(A)-RNA was separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran, Amersham), and UV-crosslinked. The RNA was hybridized to a ^{32}P-labled, NS1I-1-specific probe derived form a fragment (corresponding to positions +791 to +1745) of the original pK5 library isolate as described (Ausubel, et al., 1992, *supra*).

7.1.5. VIRUSES, CELLS, AND EXTRACTS

Influenza strains A/WSN/33 (H1N1), A/Berkeley/1/68 (H2N2), A/Beijing/32/92 (H3N2), A/duck/Alberta/76 (N12N5), A/turkey/Oregon/71 (H7N5), and B/Lee/40 were grown in the allantoic cavity of 10 days old embryonated chicken eggs. Confluent monolayers of Madin Darby canine kidney-(MDCK)-cells were infected with influenza viruses at an m.o.i. of 10 for one hour in 35 mm dishes. Infection was continued at 37°C (influenza A viruses) or 35°C (influenza B/Lee/40) for 5 hours in MEM-medium containing 0.1% bovine serum albumin. Cells were labeled with 100 µCi of ^{35}S-methionine and ^{35}S-cysteine (ICN) per dish for one hour in MEM-met$^-$cys$^-$-medium. Cells were washed and scraped in ice-cold phosphate buffered saline (PBS). Cells from one dish were lysed with 500 µl NET-N buffer (10mM Tris/HCL pH 8.0, 1 mM EDTA, 150 mM Nacl, 0.05% Nonidet P 40) and two 30 second pulses in a Raytheon sonicator at a setting of 1A. Lysates were centrifuged for 10 minutes at 20,000 rpm in a TL100.3 rotor. The supernatants were used for precipitation of proteins.

7.1.6. EXPRESSION OF GST-NS1I-1 FUSION PROTEIN IN *E. COLI* AND PRECIPITATION OF VIRAL PROTEINS FROM CELL EXTRACTS

NS1I-1 was expressed in *E. coli* BL26 from pGEX-NS1I-1 as a GST (glutathione-S-transferase)-NS1I-1 fusion protein with a predicted molecular weight of 77 kDa. Production of GST-NS1I-1 was induced using isopropyl-β-D-galactopyranoside essentially as described (Smith, et al., 1988, *supra*). GST-NS1I-1 was adsorbed from bacterial lysates to glutathione sepharose beads (Pharmacia) as recommended by the manufacturer. Beads were washed three times with PBS to remove contaminating proteins. 10 µl of glutathione sepharose coated with GST-NS1I-1 fusion protein was rotated with 100 µl extract of virus-infected MDCK-cells (see above) in 750 µl NET-100 buffer (20 mM Hepes,pH 8.0, 100mM NaCl, 0.5 mM DTT) for 90 minutes at 4°C. The beads were washed three times with PBS/0.05% NP-40 and precipitated proteins were analyzed by SDS-gel electrophoresis and autoradiography. In parallel reactions, viral proteins were immunoprecipitated from 50 µl of infected cell extracts using 5 µl of anti-NS1 or anti-M1 antiserum and protein A agarose as described (Harlow & Lane, 1988, *supra*). As a negative control, GST protein was expressed in BL26 from pGEX-5X-1 and used the same way in the co-precipitation assay.

7.2. RESULTS

7.2.1. ISOLATION OF NS1-INTERACTING FACTORS

The yeast interaction trap system (Gyuris, et al., 1993, *supra*; Zervos, et al., 1993, *supra*) was used to identify cellular proteins that interact with the non-structural protein NS1 of influenza A virus. A LexA-NS1 fusion protein was used as bait to screen

library in which HeLa cell cDNAs were expressed as fusions with an acidic transcription activation domain (Gyuris, 1993 #159). Colonies were selected, in which either of two reporter genes, LEU2 and lacZ, were activated by the cDNA-encoded proteins. This double selection scheme was used to increase the stringency, because in an initial screen a high proportion of candidates scored negative in subsequent genetic tests. The library plasmids were isolated from the selected clones.

The binding specificity of the encoded fusion proteins was tested by assaying the activation of a lacZ-reporter gene encoded on pSH18-34. Expression of β-galactosidase from this plasmid is transcriptionally controlled by LexA-specific operator sites. The isolated library plasmids were co-transformed with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses a LexA-bicoid fusion protein and was used as a non-specific operator-binding control. The resulting strains were assayed for β-galactosidase activity specifically on X-gal plates containing galactose, but not glucose. From 3.3×10^5 independent library transformants, three plasmids were isolated that induced galactose-specific activation of the lacZ reporter gene only in combination with pLexA-NS1. Sequence analysis indicated that the three plasmids were each derived from different cellular cDNAs.

7.2.2. CLONING AND SEQUENCE ANALYSIS OF NS1I-1

One of the isolated plasmids, pK5, was analyzed further. It carried a cDNA-insert of 1781 bp with an open reading frame of 1413 nucleotides followed by 368 nucleotides of a potentially untranslated region (Fig. 12). The cDNA terminated with an oligo(A)-tract and had a consensus poly(A)-

site at positions 2526-2531. Northern blot analysis of HeLa cell poly(A)-RNA using a NS1I-1-specific probe detected one single transcript of about 3.0 kb suggesting that the pK5 insert represented an incomplete cDNA (Fig. 13). The remaining NS1I-1 cDNA was cloned by a 5'RACE procedure (Frohman, et al., 1988, *supra*). Four independent clones were sequenced that differed only in length at the very 5'-end. The longest 5'RACE product, contained in pRACENS1I-1, extended the NS1I-1 sequence for 893 nucleotides upstream totalling in a cDNA of 2674 bp (Fig. 12). The sequence has one long open reading frame encoding a protein of 735 amino acids with a predicted molecular mass of 79.5 kDa and a pI of 9.06. The putative ATG-start codon is located 103 nucleotides downstream of the 5'-end and is in the context of a sequence consistent with its being a translational start (Kozak, 1989, J. Cell Biol. 108: 229-241).

Sequence comparisons through the EMBL- and Genbank databases using the FASTA- and TFASTA-analysis programs revealed that NS1I-1 is highly homologous to porcine 17β-estradiol dehydrogenase (Leenders, et al., 1994, *supra*). The two cDNAs are 86% identical on the nucleic acid level. The encoded proteins are 84% identical and are 92% similar when allowing for conserved amino acid substitutions. NS1I-1 cDNA also shows strong homology to ten human cDNA fragments that have been isolated as expressed sequence tags, as revealed by a BLAST-analysis of the NCBI-database (fragments are between 134 to 556 bp in length). These cDNAs were derived from different tissues including liver, spleen, brain, adipose tissue, and adrenals tissue indicating a broad expression of NS1I-1 in the body.

The encoded NS1I-1 protein features two conserved sequence motifs of the short-chain alcohol

dehydrogenase family (Persson, et al., 1991, Eur. J. Biochem. 200: 537-543). Specifically, amino acids 15 to 22 (TGAGAGCG) are similar to the potential co-factor binding site, and residues 163 to 167 (YSAAK) correspond to a short stretch that has been suggested to participate in catalysis (Chen, et al., 1993, Biochemistry 32: 3342-3346). The presence of the tri-peptide AKL at the carboxy-terminus was also noted. Similar tri-peptide motifs have been found to serve as targeting signals for import into microbodies (for a review, see de Hoop & Ab, 1992, Biochem. J. 286: 657-669). However, the presence of this signal does not automatically direct a protein to these organelles (de Hoop & Ab, 1992, *supra*).

7.2.3. NS1I-1 BINDS NS1 PROTEIN FROM EXTRACTS OF INFLUENZA VIRUS INFECTED CELLS

In order to confirm a physical interaction between NS1I-1 protein and NS1 expressed in influenza virus infected cells, a co-precipitation assay was performed as similarly described in Section 6.2.3, above, for NPI-1. A glutathione-S-transferase(GST)-NS1I-1 fusion gene was constructed and expressed in *E.coli*. GST-NS1I-1 fusion protein from bacterial lysate was absorbed to the affinity matrix glutathione agarose and purified from contaminating bacterial proteins. The immobilized fusion protein was used to bind and precipitate ^{35}S-labeled proteins from extracts of MDCK cells infected with human influenza A/WSN/33 viruses (Fig. 14). The NS1 protein of this strain is 98% identical to its counterpart from A/PR/8/34 used in the yeast interaction screen. Aliquots of the same extract were used to in parallel reactions to immunoprecipitate influenza virus proteins NS1 and M1. The precipitated proteins were analyzed by SDS-gel

electrophoresis and visualized by fluorography. Fig. 14 shows, that GST-NS1I-1 efficiently precipitated a protein band co-migrating with immunoprecipitated NS1 protein from infected cell extract (compare lanes 2 and 3). This interaction was specific for NS1I-1 since no proteins were detected in precipitates using GST only (lane 6). In addition, no proteins were precipitated by GST-NS1I-1 from mock-infected cells (lane 8), showing that a virus induced protein was recognized by NS1I-1. This experiment confirmed, that NS1I-1 interacts specifically with the NS1 protein of influenza A virus.

If this interaction is important for the viral life-cycle one would expect it to be conserved. Consequently, the binding of NS1I-1 to NS1 proteins from other influenza A strains should be detectable despite of their considerable variation in the primary structure (Baez, et al., 1981, Virology 113: 397-402; Ludwig, et al., 1991, Virology 183: 566-577). Therefore the interaction between NS1I-1 and NS1 was examined using the same co-precipitation assay described above, with extracts from cells infected with different influenza A and B virus strains.

Mutations accumulate in the NS1 gene at a steady rate over time (Buonagurio, et al., 1985, Science 232: 980-982). Thus, the time-span between the isolation of two strains is reflected in the sequence variation of its NS1 proteins (Ludwig, et al., 1991, *supra*; Buonagurio, et al., 1985, *supra*). NS1I-1 binding to NS1 proteins from two recently isolated human influenza A strains A/Beijing/32/92 and A/Berkeley/1/68 was examined. As can be seen in Fig. 15, Panels C and D, respectively, NS1 proteins from both strains were specifically precipitated (Fig 15, Panels C and D, lanes "GST-K5"). A low immunoprecipitation efficiency of NS1 protein from the

Beijing-strain (Panel C) was reproducibly observed. The NS1 proteins of A/Berkeley/1/68 and A/WSN/33 are 90.8% identical to each other. The NS1 sequence of A/Beijing/32/92 is not known.

The following analyses were conducted to examine whether GST-NS1I-1 is also recognized by the more divergent NS1 proteins of the avian influenza strains A/duck/Alberta/76 and A/turkey/Oregon/71. The NS1 proteins of these strains are 66.5% and 63.6% identical, respectively, to A/WSN/33. Significantly, NS1 of A/turkey/Oregon/71 is only 124 amino acids in length, lacking most of the carboxy-terminal half of other NS1 proteins, which consist of 207 to 237 amino acids (Norton, et al., 1987, Virology 156: 204-213). Nevertheless, precipitation of a protein band co-migrating with NS1 from both strains was observed (Fig. 15, Panels A and B, lanes "GST-K5"). The NS1 and M1 proteins of A/duck/Alberta/76 could not be separated by the gel system used. Significant amounts of nucleoprotein in the GST-NS1I-1 precipitates of these avian strains were reproducibly detected for undetermined reasons.

Finally, the co-precipitation assay was used to test the human influenza B virus B/Lee/40. Surprisingly, GST-NS1I-1 precipitated specifically the influenza B virus NS1 protein, although it is only 20.6% identical to NS1 from A/WSN/33 (Fig. 15, Panel E, lane "GST-K5"). Taken together, the binding of GST-NS1I-1 to NS1 proteins expressed by several influenza A and B virus stains could be demonstrated, despite the great divergence of their primary structures. This result strongly supports an important function of this interaction during the viral life cycle, and indicates that the NS1I-1 interaction is an excellent target for antiviral intervention.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.